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14. ABSTRACT
We showed that the human reticulocytes made in our culture system have a hemoglobin concentration and types and major membrane proteins are identical to those in adult human red cells; these reticulocytes are as stable when transfused into NOD-SCID immune deficient mice as are normal human red cells, We completed an additional screen of 30 compounds known to activate or inhibit nuclear receptors other than the glucocorticoid receptor, and showed that two PPARα receptor agonists, fenofibrate and GW7647, potently synergized with dexamethasone in stimulating self-renewal of human and murine BFU-E progenitors and in stimulating 3- fold the output of human red cells in culture. We showed that knocking down expression of rps19 at the end of the initial expansion phase of our CD34+ cell culture system inhibited cell proliferation about 10 – fold, as expected, and that GW7647 stimulated red cell production in these knockdown cells ~3- fold. We also showed that the clinically tested Amgen prolyl hydroxylase inhibitor stimulated red cell production in cultures of human CD34+ stem/progenitor cells at extremely low concentrations, and that GW7647 synergized with the Amgen prolyl hydroxylase inhibitor to provide additional stimulation of red cell production in these cultures; this suggests that a combination of these two FDA- approved drugs could be used to treat certain bone marrow failure disorders. We showed that the PPARα agonist GW7647 stimulates red cell formation in "Nan" (neonatal anemia) mice, raising the level of red cells to almost normal. It also causes an increase in the numbers of splenic BFU-E progenitors, suggesting that GW7647 increases erythroid output via promoting BFU-E self-renewal.

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Introduction:

Red blood cell levels are normally tightly regulated by erythropoietin (Epo), and anemia associated with chronic renal insufficiency and malignancy can be successfully treated with recombinant Epo [1]. Epo stimulates erythropoiesis by promoting survival, proliferation, and terminal differentiation of colony-forming unit Erythroid (CFU-E) cells; over 3- 5 days each CFU-E will produce ~10- 60 mature enucleated erythroid cells [2-5]. Since normal Epo levels are very low, red cell output from CFU-E cells can be increased more than one order of magnitude by increased Epo production or by injection of recombinant Epo. However because each CFU-E cell can undergo only 3-6 terminal cell divisions under maximum Epo-stimulation, the number of CFU-E cells will eventually limit the response to Epo; the number of CFU-Es therefore determines maximum Epo-dependent erythrocyte output.

Many forms of acute and chronic anemia are not treatable with erythropoietin (Epo) because the CFU-E erythroid progenitors that respond to Epo are either too few in number or are not sensitive enough to Epo to maintain adequate red blood cell production. Treatment of Epo-resistant anemias requires a drug that acts earlier than Epo in the pathway of red cell formation, and that enhances the formation of the Epo-sensitive CFU-E red blood cell progenitors rather than mimicking Epo in stimulating the terminal proliferation and differentiation of these progenitor cells. No such therapy exists today, but research we have conducted in the past, coupled with our new work described in this report, points the way to several types of small molecule drugs that, individually or in combination, can potentially be used to expand erythroid progenitors and to treat these disorders.

In situations where the maximal Epo-dependent output is not sufficient (hemolysis, sepsis, genetic bone marrow failure diseases such as Diamond Blackfan anemia, or severe trauma), the organism attempts to increase the number of Epo-responsive cells through a mechanism known as stress erythropoiesis (SE). [6,7] Unlike steady-state erythropoiesis, which is largely regulated by Epo, SE requires other factors such as stem cell factor (SCF) and glucocorticoids (GCs) [6-8]. The therapeutic effect of the corticosteroid prednisone in patients with the red cell progenitor disorder Diamond-Blackfan Anemia (DBA) is well documented, though severe side effects limit its use [9,10].

In 2011 we reported that glucocorticoids stimulate self-renewal of early Epo-independent progenitor cells (burst-forming units erythroid or BFU-Es), over time increasing production of colony-forming units erythroid (CFU-E) erythroid progenitors from the BFU-E cells, and enhancing the numbers of terminally differentiated red cells. GCs do not affect proliferation of CFU-E cells or erythroblasts. In mRNA-seq experiments, we found that glucocorticoids induced expression of ~86 genes more than 2-fold in BFU-E cells. Computational analyses indicated that, of all transcription factors, binding sites for hypoxia-induced factor 1 alpha (HIF1α) were most enriched in the promoter regions of these genes, suggesting that activation of HIF1α may enhance or replace the effect of glucocorticoids on BFU-E self-renewal. Indeed, HIF1α activation by the pan-prolyl hydroxylase inhibitor (PHI) DMOG synergized with glucocorticoids and enhanced production of CFU-Es and later erythroblasts over 170-fold. PHI-induced stimulation of BFU-E progenitors thus represents a conceptually new therapeutic window for treating Epo-resistant anemia. We proposed a physiological model of stress erythropoiesis where increased levels of GCs –systemic stress hormones - and reduced oxygen – local stress - help maintain the earliest erythroid progenitors, increase CFU-E output, and at the same time stimulate terminal differentiation, thus promoting both a rapid and long-lasting increase in red blood cell production [11].

These studies led us to generate two hypotheses that underlie the research supported by this grant.

First, we proposed that combinations of specific prolyl hydroxylase inhibitors, those that indirectly activate the transcription factor HIF-1α, and also low concentrations of certain glucocorticoid receptor agonists or partial agonists, can significantly expand mouse bone marrow and human blood erythroid progenitors in culture. We hypothesized that this novel combination of chemical activators will stimulate the terminal proliferation and differentiation of these progenitor cells and lead to enhanced production of red blood cells without the side effects of prolonged corticosteroid therapy.

Second, we hypothesized that small molecule drugs already approved for other indications might also be able to stimulate BFU-E self-renewal and thus serve as additional therapeutic agents that could increase output of red blood cells and treat anemias untreatable by erythropoietin. Thus we proposed to use our mouse fetal liver BFU-E culture system to screen a library of ~2000 tested and approved therapeutic and other compounds for novel molecules that can stimulate red cell production in culture, either at the BFU-E or CFU-E level.

Our immediate aim is to provide new and improved treatments for young children with Diamond Blackfan Anemia and other bone marrow failure disorders. Our work will likely lead to treatments for several types of Epo resistant anemias that affect our active duty personnel as well as our veterans, including kidney dialysis patients, patients with trauma or sepsis, and possibly anemia associated with malaria.

BODY

This report covers work done on the project through 8/31/14, no further research was conducted during the No Cost Extension period of 9/1/14-8/31/15 since no additional funds were awarded to pay for the continuation of the project. Below is a summary of the work concluded.

1. **Characterization of human reticulocytes synthesized in culture.**

In this study human mobilized CD34+ blood cells were obtained from the Fred Hutchinson Cancer Center. These CD34+ cells were provided by the Fred Hutchinson's Core facility in a completely de-identified manner (they are assigned an arbitrary research code number on the label), and they are procured under the Core's IRB-approved research collection protocol and research repository operation that prevents the Core from releasing any specific donor identification information to the end user other than generic aspects such as age and gender. In addition, these CD34+ cells are not collected specifically for the currently proposed research project through an interaction with living individuals and the investigator cannot ascertain the identity of the individual(s) from whom the cells were derived.

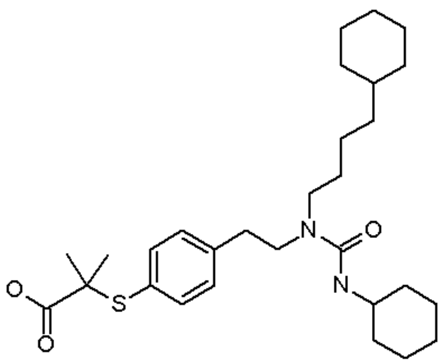
Therefore these cells do not meet the definition of human subjects research under HHS regulations at 45 CFR Part 46 because (1) the cells were not collected specifically for the currently proposed research project through an interaction or intervention with living individuals; and (2) the investigator cannot readily ascertain the identity of the individual(s) to whom the coded private information or specimens pertain because there are IRB-approved written policies and operating procedures for a repository that prohibit the release of the key to the investigators under any circumstances.

As detailed in our 2013 Annual Report we developed a four stage 22-day culture of these human cells resulting in a synchronous 20,000 fold expansion. Over half of the cells underwent enucleation; the resulting reticulocytes have the normal amount of hemoglobin – 30 pg/cell- characteristic of human red cells and also have the same diameter of ~7 μM. We showed that over half of the protein in these cells is hemoglobin A, and that the hemoglobin composition is identical to that of normal human red blood cells. That the cultured reticulocytes have more non-globin protein than red cells is expected as reticulocytes degrade or exocytose all cytosolic proteins not destined for incorporation into mature red cells. Additionally, the composition of the major membrane proteins in these reticulocytes is the same as that of normal red blood cells, including spectrin, ankyrin, Band 3, and the cytoskeletal proteins 4.1, 4.2, and actin.

A preliminary experiment showed that these cultured reticulocytes survive normally in immune compromised mice. In this study 2 - 5 x10^8 normal human red blood cells or cultured CD34+ cells at the end of differentiation (> 50% enucleated) were labeled with CFSE, a green fluorescent dye that stably labels the cell plasma membrane, and injected to NOD-SCID mice 6 - 8 weeks of age. Blood was analyzed 3 days after injection. The number of cultured red cells surviving (1.5% of total blood cells, most of which of course were endogenous mouse red cells) was comparable to that of normal human red blood cells; ongoing studies will quantify the survival of these cells in mice.

2. Screening of additional compound libraries identified agonists of the PPARa receptor that synergize with very low concentrations of the corticosteroid dexamethasone to increase the erythroid output of murine and human erythroid progenitors.

We screened **a** the library of 30 nuclear receptor agonists and antagonists, using the same protocol we employed previously to screen the library of >2000 tested and approved therapeutic compounds. There were several "hits," but the most effective was the PPARα receptor agonist GW7647 originally developed by GlaxoSmithKline for dyslipidemia. It is a potent and highly selective PPARα agonist (EC50 values are 6, 1100 and 6200 nM for human PPARα, PPARγ and PPARδ receptors respectively). It exerts cardioprotective effects in a mouse model of acute ischemia/reperfusion myocardial injury and has lipid-lowering effects following oral administration *in vivo.*



a. In vitro cultures In expansion cultures of purified murine BFU-E cells GW7647 synergized with 100 µM dexamethasone in stimulating the production of erythroid cells, resulting in a greater than 10-fold increase in red cell numbers. GW7647 also synergized with even lower concentrations of dexamethasone in stimulating production of red cells in culture from mouse BFU-E cells. This is important since one goal for new treatments of Diamond Blackfan Anemia is to use low dosages of prednisone (a corticosteroid) together with a drug like GW7647 that synergizes with it stimulating red cell formation without the side effects of corticosteroids.

As evidenced by increased formation of BFU-E progenitors (quantified by colony assays) during culture, GW7647 synergized with 100 nM dexamethasone in stimulating self renewal of murine BFU-E cells. The morphologies of these BFU-E colonies formed from cultures containing dexamethasone and the PPARa receptor agonist were of normal size. Additionally, fenofibrate, another but less selective clinically- approved PPARα agonist, functions similar to GW7647 in stimulating BFU-E self renewal.

GW7647 also synergized with100 nM dexamethasone in stimulating formation of human red cells in culture of human CD34+ stem/ progenitor cells; the output of red cells at 22 days of cultures containing both 100 nM dexamethasone and GW7647 was about three fold that of cultures containing 100 nM dexamethasone alone. Consistent with the results from mouse cultures, GW7647 also synergized with 100 nM dexamethasone in stimulating self-renewal of human BFU-E cells.

We previously reported that a pan-specific prolyl hydroxylase inhibitor DMOG synergized with dexamethasone to stimulate BFU-E self-renewal and increase the output of human erythroid cells in culture. Recently we reported that the clinically tested Amgen prolyl hydroxylase inhibitor also stimulated red cell production in cultures of human CD34+ stem/progenitor cells at the extremely low concentration of 10^{-10} M. Importantly, GW7647 synergized with 10^{-10} M of the Amgen prolyl hydroxylase inhibitor to provide additional stimulation of red cell production in these cultures; this suggests that a combination of these two FDA- approved drugs could be used to treat certain bone marrow failure disorders such as Diamond-Blackfan anemia.

b. Human cell cultures modeling Diamond-Blackfan anemia. We focused on developing additional human cell culture models that can be used to test potential drugs, such as cimetidine and GW7647, for their ability to treat Diamond Blackfan anemia and other bone marrow failure disorders.

We experimented with several protocols for knocking down expression of rps19 and other ribosomal proteins in our cell culture system, one that supports synchronized expansion, terminal differentiation, and enucleation of mobilized human blood CD34+ stem and progenitor cells. Knocking down expression of rps19 at the beginning of the initial 4- day expansion period caused considerable cell death; nonetheless GW7647 stimulated red cell production several fold.

Knocking down expression of rps19 at the end of the initial expansion phase – the beginning of the First Differentiation Stage (Diff I) - inhibited cell proliferation about 10 – fold, as expected. Importantly, GW7647 stimulated red cell production in these knockdown cells, resulting in a ~3- fold increase in erythroid cells at the end of the culture period. Addition of GW7647 stimulated production of both BFU-E and CFU-E progenitors 2 – 3 fold early in the culture period. We are now testing whether, as we expect, fenofibrate and cimetidine can also synergize with the corticosteroid dexamethasone in stimulating production of human red cells in this system. We are also using similar lentiviral systems to knock down expression of other ribosomal proteins in these cultures, and testing the effects of these drugs in these cultures.

c. Mouse models of Epo- resistant anemias Glucocorticoids and the glucocorticoid receptor are required for erythroid lineage cell expansion in the spleen during stress erythropoiesis. Using a phenylhydrazine (PHZ)-induced hemolytic anemia mouse model, we showed that that GW7647 stimulates stress-induced erythroid lineage expansion *in vivo*. In this preliminary study the mice were pre-treated with GW7647 or DMSO (control) for 3 days before the PHZ injection, and the treatment was continued for another 8 days. Importantly, the drop in hematocrit from the basal level of ~50% and the initial drop in red cell numbers and hemoglobin levels was reduced by injection of GW7647. Additional evidence that GW7647 stimulates red cell formation *in vivo* was the increased level of reticulocytes in the blood of GW7647- treated mice relative to the control PHZ- injected mice. The levels of platelets and granulocytes was unaffected by GW7647 treatment.

We have imported Nan/+ mice from Dr. Peters of the Jackson Laboratory and are breeding them in our animal facility. The mutant "Nan" (neonatal anemia) mouse has a single amino acid substitution (E339D) in the second zinc finger of the erythroid- important transcription factor EKLF, which abrogates DNA-binding capacity of EKLF to certain target genes. While Nan/Nan homozygotes are embryonically lethal, heterozygotes (Nan/+) survive with a life-long, intermediate- to- severe hemolytic anemia, displaying many features of hereditary spherocytosis (Slatecka et. al. 2010 PNAS 107:15151). We have repeated experiments showing that the PPARα agonist GW7647 stimulates red cell formation in these mice, raising the level of red cells to almost normal. It also causes an increase in the numbers of splenic BFU-E progenitors, suggesting that as expected GW7647 increases erythroid output via promoting BFU-E self-renewal. These mice will also be used to test the histamine H2 receptor inverse agonists and other drug candidates.

KEY RESEARCH ACCOMPLISHMENTS:

• We characterized the human reticulocytes made in our culture system. We showed that their hemoglobin concentration and types and major membrane proteins are identical to those in adult human red cells. We showed these reticulocytes are stable for several days when transfused into NOD-SCID immune deficient mice, equivalent to survival of normal human red cells,

• We completed an additional screen of 30 compounds known to activate or inhibit nuclear receptors other than the glucocorticoid receptor. We identified several "hits," and showed that two potently synergized with dexamethasone in stimulating self-renewal of human and murine BFU-E progenitors and in stimulating 3- fold the output of human red cells in culture. These PPARα receptor agonists, fenofibrate and GW7647, originally developed by for dyslipidemia, can be used in clinical trials for treatment of anemias such as Diamond Blackfan Anemia that do not respond to erythropoietin treatment.

• We showed that knocking down expression of rps19 at the end of the initial expansion phase of our CD34+ cell culture system inhibited cell proliferation about 10 – fold, as expected. Importantly, GW7647 stimulated red cell production in these knockdown cells ~3- fold. Addition of GW7647 stimulated production of both BFU-E and CFU-E progenitors early in the culture period, consistent with the notion that this drug stimulates self-renewal of human DBA BFU-E erythroid progenitors.

• We showed that the clinically tested Amgen prolyl hydroxylase inhibitor stimulated red cell production in cultures of human CD34+ stem/progenitor cells at extremely low concentrations, and that GW7647 synergized with the Amgen prolyl hydroxylase inhibitor to provide additional stimulation of red cell production in these cultures; this suggests that a combination of these two FDA- approved drugs could be used to treat certain bone marrow failure disorders.

• We showed that the PPARα agonist GW7647 stimulates red cell formation in "Nan" (neonatal anemia) mice, raising the level of red cells to almost normal. It also causes an increase in the numbers of splenic BFU-E progenitors, suggesting that GW7647 increases erythroid output via promoting BFU-E self-renewal. These mice will also be used to test the histamine H2 receptor inverse agonists and other drug candidates.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

manuscripts, abstracts, presentations. None at this time.

licenses applied for and/or issued. None yet and we do not plan to apply for use patents for many of the molecules we identified that stimulate red cell formation. We want physicians to be free to use the compounds to treat patients and others to modify or improve o0ur protocols.

degrees obtained that are supported by this award None during this period.

development of cell lines, tissue or serum repositories None anticipated.

informatics such as databases and animal models, etc. None during this period.

funding applied for based on work supported by this award No additional funding at this time.

employment or research opportunities applied for and/or received based on experience/training supported by this award. None this year

CONCLUSION: For the last reporting period, 9/1/2014-8/31/2015, no further research goals were attained due to the fact that no additional funds were awarded during this period. We had requested a No Cost Extension, which we received, but no additional funds were forthcoming so we abandoned the project. Overall, during the period 9/1/2012 -8/31/2014, our cell culture assays identified several drugs approved by the FDA for other indications that could be repurposed as potential treatments for bone marrow failure disorders such as Diamond Blackfan anemia, kidney dialysis patients, patients with trauma or sepsis, and possibly anemia associated with malaria. There are currently no acceptable therapies for these disorders. We needed to continue to characterize the red cells produced in these cultures to confirm that they were normal, and to test them in additional cell culture and animal models of these human diseases. We believed that several of these drugs could enter clinical trials for these diseases within a year or two since their safety in humans has already been validated extensively. However, since no funds were made available, these studies were not carried out.

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